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## Role of GABAA receptor subtypes in the behavioural effects of intravenous general anaesthetics

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**The role of specific GABA<sub>A</sub> receptor subtypes in mediating the behavioural effects of intravenous general anaesthetics.**

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The role of specific GABA<sub>A</sub> receptor subtypes in ~~mediating~~ the behavioural effects of intravenous general anaesthetics.

Running title: Mechanisms of intravenous general anaesthetics

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### Summary

Since the introduction of general anaesthetics into clinical practice researchers have been mystified as to how these chemically disparate drugs act to produce their dramatic effects on central nervous system function and behaviour. Scientific advances, particularly during the last 25 years ~~or so~~, have now ~~finally~~ begun to reveal the molecular mechanisms underpinning their behavioural effects. For certain intravenous general anaesthetics such as etomidate and propofol a persuasive case can now be made that the GABA<sub>A</sub> receptor, a major inhibitory receptor in the mammalian central nervous system, is an important target. ~~Furthermore,~~ ~~a~~Advances in molecular ~~biology-pharmacology~~ and in genetic manipulations of rodent genes reveal that different subtypes of the GABA<sub>A</sub> receptor are responsible for mediating particular aspects of the anaesthetic behavioural repertoire. Such studies ~~are permitting~~provide a better understanding of the neuronal circuitry involved in the various anaesthetic-induced behaviours and in the future may result in the development of novel therapeutics with a reduced propensity for side-effects.


### Key Words:

*Anaesthetics i.v., etomidate;*

*Anaesthetics i.v., propofol;*

*Receptors, GABA<sub>A</sub>*

The discovery over 170 years ago of agents ~~over 170 years ago~~ that when administered produced a state of reversible unconsciousness, revolutionised surgery and remains one of the most important medical innovations. Although during the last century the clinical use of general anaesthetics became widespread, the molecular mechanism(s) whereby they produced their remarkable repertoire of behavioural effects, which includes sedation, immobility, amnesia and unconsciousness, remained a mystery. Agents capable of inducing a state of general anaesthesia are chemically diverse (Figure 1). ~~T and this~~ variety-chemical diversity appeared to preclude involvement of a common molecular anaesthetic target, e.g. a classical drug receptor interaction, as no obvious structure-activity relationship was evident<sup>1-5</sup>. Such diversity led early theories of general anaesthetic activity to focus on non-specific interactions within the central nervous system (CNS). Over a century ago ~~For example,~~ Meyer and Overton reported a striking correlation between the oil:water partition coefficients of a range of anaesthetic compounds and their ability to immobilise tadpoles. ~~i.e. the~~ The more lipid soluble the compound, the greater is ~~their~~ anaesthetic potency. Based on these observations it was subsequently proposed that general anaesthetics perturb lipid bilayers to induce ~~a~~ 'non-specific' disruption of neuronal activity<sup>6</sup>. As exceptions to this correlation began to emerge, the concept that particular proteins may-might represent viable anaesthetic targets gained traction.

An important demonstration that proteins had the potential to be relevant anaesthetic targets resulted from studies by Franks & Lieb<sup>7</sup>. They demonstrated that the activity of a soluble protein, firefly luciferase, prep  in the absence of lipid, was influenced by a variety of general anaesthetics<sup>7</sup>. Subsequently, numerous candidate proteins were proposed as putative targets. However, before a protein can be considered as a plausible target certain criteria need to be met<sup>8</sup>: the protein should be sensitive to clinically relevant concentrations of the anaesthetic; (although the accurate determination of behaviourally appropriate concentrations in the CNS may-can be problematic); the protein must be expressed at appropriate anatomical sites within the CNS, and, if the anaesthetic exhibits stereo-selective activity, this specificity should ideally be mirrored both by the putative target and in behavioural studies. Based on these criteria, certain transmitter-gated ion channels (TGICs) emerged as putative targets for intravenous general anaesthetics<sup>9</sup>. For an account of ~~of~~ the mechanisms of inhalational anaesthetics see<sup>10</sup>.


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Here we ~~will~~ review ~~the~~ evidence that the GABA<sub>A</sub> receptor (GABA<sub>A</sub>R) is an important target for certain intravenous general anaesthetics (Figure 1), and describe the progress being made in elucidating which GABA<sub>A</sub>R subtypes mediate the constellation of behaviours produced by these drugs. Such studies are revealing important information on the influence of anaesthetics on the neuronal circuitry associated with sedation, unconsciousness, analgesia and cognition, and in the future ~~may-might~~ lead to ~~the~~ development of improved therapeutics.

#### *Transmitter-gated ion channels as targets for intravenous general anaesthetics.*

The TGICs considered here are members of one of two genetically distinct receptor super-families. The 'cys-loop' TGICs include the GABA<sub>A</sub>R<sub>s</sub> (Figure 1), ~~the~~ strychnine-sensitive glycine receptors, ~~the~~ nicotinic acetylcholine receptors (nAChR) and ~~the~~ 5-hydroxytryptamine 3 receptors (5-HT3R)<sup>11</sup>. In common, these cys-loop receptors are assembled from five transmembrane crossing subunits, arranged to form an integral ion channel pore (Figure 1). The glutamate-gated ion channels (including AMPA, NMDA and kainate receptors), exhibit a distinct membrane topology from the "cys-loop" family and are composed of four transmembrane crossing subunits, again arranged to form an integral ion channel pore<sup>11</sup>. For all TGICs, receptor activation by ~~the~~ neurotransmitter produces a rapid conformational change ~~of the protein,~~ causing the associated ion channel pore to open, permitting ~~the~~ selective movement of anions (for GABA<sub>A</sub>R & glycine receptors), or cations (~~for~~ AMPA, NMDA, kainate, nAChR and 5HT3R) across the ~~neuronal~~ membrane<sup>11</sup>. The net flow of either cations, or anions, ~~will~~ depends upon the neuronal membrane potential and the relative intracellular and extracellular concentrations of the ionic species. ~~Usually,~~ ~~R~~ receptor activation of cation- ~~and-or~~ anion-associated channels ~~will-usually~~ results in neuronal depolarisation, or hyperpolarisation, respectively.


~~Simplistically~~ Overall, general anaesthetics ~~mi~~  act to enhance the function of inhibitory receptors (GABA<sub>A</sub>R<sub>s</sub> or glycine receptors), or inhibit ~~their~~ excitatory ~~counterparts~~ receptors (nicotinic, 5HT3 and ionotropic glutamate receptors). However, it is important to establish which members of the TGIC family are behaviourally relevant targets for intravenous general anaesthetics, and to determine whether all anaesthetics exhibit a common receptor profile. In the 1980s and 1990s a number of ~~laboratories-investigators~~ demonstrated ~~that~~ the function of ~~the~~ GABA<sub>A</sub>R<sub>s</sub> ~~to-bewas~~ enhanced by anaesthetic barbiturates, alphaxalone, etomidate and propofol, but few studies examined their relative selectivity for GABA<sub>A</sub>R<sub>s</sub> versus other TGICs.

when determined under identical recording conditions<sup>9 12–15</sup>. We therefore used the voltage-clamp technique to investigate the interaction of four structurally distinct intravenous general anaesthetics (etomidate, propofol, alphaxalone and pentobarbitalone) with a variety of recombinant TGICs expressed in *Xenopus laevis* oocytes (Figure 2). The receptors investigated included representatives of the major inhibitory anion conducting receptors in the CNS (GABA<sub>A</sub>R<sub>s</sub> and glycine receptors), members of the major excitatory glutamate-gated cation channels (AMPA and NMDA receptors), together with the cation-conducting nAChR and the 5HT<sub>3</sub>R. This profiling revealed all four anaesthetics to act as positive allosteric modulators (PAMs) of the GABA<sub>A</sub>R<sub>s</sub>, i.e. at behaviourally-relevant concentrations in that they enhanced the response of the receptor to GABA (Figure 2). Additionally, at greater concentrations, in common all of these anaesthetics directly activated the GABA<sub>A</sub>R<sub>s</sub> in the absence of GABA (i.e. a “GABA-mimetic” effect). Etomidate was of particular interest as at low  $\mu$ M–micromolar concentrations it enhanced GABA-evoked responses, but had no effect on the genetically closely related anion-conducting glycine receptor, or on any of the cation-conducting receptors (Figure 2). Similarly, propofol and alphaxalone were relatively selective for the GABA<sub>A</sub>R, although they additionally exhibited a also modestly inhibitory effect upon neuronal nicotinic receptors (Figure 2). An effect on nicotinic receptors is unlikely to be crucial to their anaesthetic activity because betaxalone, the behaviourally inert 3 $\beta$ -ol isomer of alphaxalone, also inhibited nicotinic receptors, but distinct from alphaxalone, it had no effect on GABA<sub>A</sub>R<sub>s</sub><sup>14 15</sup>. Although pentobarbitalone was rather non-selective, it too enhanced GABA<sub>A</sub>R<sub>s</sub> function (Figure 2). Therefore, in conclusion this comparative study, together with complementary studies, identified the GABA<sub>A</sub>R as represent a viable target for these intravenous general anaesthetics. Note however, that not all intravenous agents-anaesthetics enhance GABA<sub>A</sub>R function, an important exception being the dissociative anaesthetic ketamine, where for which the NMDA receptor is implicated<sup>10 16</sup>.

Given the this receptor selectivity profile outlined above, the actions of etomidate warranted further investigation. In contrast to most clinical anaesthetics, etomidate is used as the resolved R-(+)-enantiomer, rather than as a racemate<sup>17</sup>. Enantioselectivity, provides a powerful tool to identify anaesthetic-relevant molecular targets<sup>18</sup>. The S-(-)-enantiomer of etomidate was less effective than the R-(+)-enantiomer in causing the loss of the righting reflex (a surrogate measurement that correlates with a loss of response to a verbal command in humans,<sup>10</sup> in both mice and tadpoles<sup>19</sup>. In agreement, the potency and the efficacy of etomidate acting on recombinant GABA<sub>A</sub>R<sub>s</sub> was far greater for the R-(+)-enantiomer, than for the S-(-)-enantiomer<sup>19</sup>


<sup>20</sup>.

### *The structure and pharmacology of ~~the~~ GABA<sub>A</sub>Rs:*


The GABA<sub>A</sub>R ~~is composed of~~ structure consists of five subunits arranged around a central anion conducting pore (Figure 1). Mammals possess a considerable repertoire of subunits ( $\alpha$ 1-6,  $\beta$ 1-3,  $\gamma$ 1-3,  $\delta$ ,  $\epsilon$ ,  $\pi$ ,  $\rho$ 1-3), which display a distinctive expression pattern in the CNS<sup>21-23</sup>. This subunit ~~palette~~  underpins the expression of 20-30 distinct GABA<sub>A</sub>R isoforms, which exhibit distinct physiological and pharmacological properties, and ~~not surprisingly,~~ given their distinctive distribution in the CNS, ~~they~~ influence different behaviours ~~as well~~ (see below). The majority of GABA<sub>A</sub>Rs contain two  $\alpha$  and two  $\beta$  subunits, together with a single ~~copy of the~~  $\gamma$ 2 subunit<sup>21-23</sup> (Figure 1). Synaptic receptors often contain the  $\gamma$ 2 subunit, although receptors incorporating this subunit ~~may can also~~ be located outside ~~with~~ the synapse. The synaptic receptors mediate fast phasic inhibition in response to a transient increase ~~of in~~ neurotransmitter resulting from the vesicular release of GABA (Figure 3). Receptors incorporating the  $\delta$  subunit, in place of the  $\gamma$ 2 subunit, are expressed extra- or peri-synaptically, and are ~~repetitively~~ activated by ambient concentrations of GABA, thereby producing a persistent form of tonic inhibition (see Figure 3 and below for further details on phasic and tonic inhibition)<sup>24-27</sup>.


The GABA<sub>A</sub>R is an important clinical target for benzodiazepines such as midazolam and diazepam, ~~where upon which~~ they act ~~as PAMs~~ to enhance the interaction of GABA with the receptor. The binding site for such drugs ~~occurs is~~ at the interface of an  $\alpha$  subunit and a  $\gamma$  subunit, usually the  $\gamma$ 2 subunit<sup>23</sup>. Recombinant GABA<sub>A</sub>Rs containing the  $\gamma$ 2 subunit, partnered with the  $\alpha$ 4, or the  $\alpha$ 6 subunit, are insensitive to benzodiazepines such as diazepam, whereas  $\alpha$ 1-,  $\alpha$ 2-,  $\alpha$ 3- and  $\alpha$ 5- $\gamma$ 2 receptors are sensitive<sup>23, 28</sup>. The molecular basis of this  $\alpha$ -subunit selective pharmacology was revealed by the construction of chimeric constructs of the  $\alpha$ 1 and the  $\alpha$ 6 subunit, leading to the identification of a critical single amino acid residue located in the N-terminal portion of the  $\alpha$  subunit<sup>23, 28</sup>. This amino acid is a histidine (H) residue for ~~the~~  $\alpha$ 1-3 and  $\alpha$ 5 subunits and an arginine (R) residue for the insensitive  $\alpha$ 4 and  $\alpha$ 6 subunits. Importantly, an H to R residue exchange by site-directed mutagenesis results in diazepam-insensitivity of the receptor. This finding ~~led to the enabled~~ creation of “knock-in” mice where ~~by~~ the  $\alpha$ 1,  $\alpha$ 2,  $\alpha$ 3, or  $\alpha$ 5 subunit was replaced with a mutant subunit incorporating the H to R mutation. Such mice have been invaluable in discovering which GABA<sub>A</sub>R subtypes mediate the behavioural repertoire produced by benzodiazepines. For example, for the  $\alpha$ 1H101R mouse the sedative action of diazepam was abolished, but the anxiolytic action ~~of this benzodiazepine~~ was




maintained<sup>23</sup>. By contrast, for the equivalent  $\alpha 2H101R$  mouse, the anxiolytic effect of diazepam was abolished, but the sedative effects remained intact<sup>23 29</sup>. These findings have encouraged the development of GABA<sub>A</sub>R-isoform selective drugs ~~e.g.~~ in the quest for a non-sedative anxiolytic<sup>23 30–32</sup>. Additionally, ligands acting as selective ~~PAMs~~  vators of spinal cord  $\alpha 2\beta 2$  GABA<sub>A</sub>Rs show promise as analgesics<sup>33–35</sup> (~~See~~ Table 1).

#### *Investigating the GABA<sub>A</sub>R isoform selectivity of intravenous general anaesthetics.*

If GABA<sub>A</sub>Rs are an important target for general anaesthetics such as etomidate, then which GABA<sub>A</sub>Rs mediate the constellation of behaviours that constitute the “anaesthetic state?” ~~Unfortunately, u~~Unlike the benzodiazepines, ~~the~~ intravenous general anaesthetics such as propofol, or the steroidal anaesthetics exhibit little, or no selectivity, for the different GABA<sub>A</sub>R subtypes. A notable exception is etomidate. ~~Our v~~Voltage-clamp studies of *Xenopus laevis* oocytes expressing human GABA<sub>A</sub>Rs, revealed etomidate to selectively enhance GABA responses mediated by activation of GABA<sub>A</sub>Rs incorporating the  $\beta 2$ , or the  $\beta 3$  subunit, but to have a reduced effect on equivalent GABA<sub>A</sub>Rs containing the  $\beta 1$  subunit<sup>36</sup>. ~~We therefore employed a similar strategy to that used to elucidate the molecular basis of the benzodiazepine  $\alpha$ -subunit selectivity. The C-~~construction of chimeric  $\beta 1$  and  $\beta 2$  subunits revealed the subunit specificity of etomidate to reside with ~~the nature of~~ a single amino acid (asparagine [N] for both the  $\beta 2$  and  $\beta 3$  subunit and serine [S] for the  $\beta 1$  subunit), located within the second transmembrane (TM2) region (a part of the protein that contributes to the lining of the ~~associated~~ anion-conducting ion ~~channel~~ pore)<sup>37</sup>. Site-directed mutagenesis of ~~the~~   $\beta 2N265S$  subunit reduced the GABA-modulatory and GABA-mimetic actions of etomidate, whereas the complementary mutation of the  $\beta 1$  residue ( $\beta 1S265N$ ) enhanced these actions of etomidate<sup>37</sup>. A methionine (M) residue occupies the equivalent position of the *Drosophila* invertebrate GABA subunit<sup>38</sup>. Mutation ~~of the to~~  $\beta 2N265M$  not only abolished the actions of etomidate, but additionally reduced the effects of propofol<sup>9 39 40</sup>.

Considering the long-held view that general anaesthetics act in a ~~rather~~ non-specific way to disrupt the neuronal membrane, these findings, at least for etomidate, were surprising. To summarise, etomidate is a highly selective ~~PAM~~  dulator of GABA<sub>A</sub>Rs, with little, or no effect at behaviourally relevant concentrations on other transmitter-gated ion channels. The anaesthetic effects of etomidate are enantioselective, a specificity that is mirrored in their interaction with ~~the~~ GABA<sub>A</sub>Rs, providing confidence that this receptor ~~may be~~ is a relevant

target. Furthermore, etomidate exhibits a clear selectivity for GABA<sub>A</sub>Rs that contain particular isoforms of the  $\beta$  subunit, a specificity that is dictated by the nature of a single amino acid of the two thousand or so that make up this pentameric receptor. Whether this residue, together with spatially related residues, contributes s\_towards an anaesthetic binding pocket is the subject of current investigation. The use of photo-labelled anaesthetic analogues of etomidate and propofol, substituted cysteine modification protection (SCAMP) techniques and molecular modelling studies, are assisting in the identification of potential binding sites within, or between selective GABA<sub>A</sub>R subunits for these intravenous anaesthetics<sup>41–43</sup>.

Although collectively these studies identify the GABA<sub>A</sub>R as a putative target for mediating the behavioural effects of etomidate, supporting *in vivo* studies are required. In this regard an important advance was made by the development of the  $\beta$ 2N265S and the  $\beta$ 3N265M knock-in mice. T.h.e. the introduction of hese mutations, which *in vitro* studies reveal to suppress the GABA<sub>A</sub>R actions of etomidate,<sup>10 44–46</sup> have been introduce  mice through genetic engineering. In the  $\beta$ 2N265S mouse the sedative actions of etomidate, as assessed by an activity box, were blunted, together with the effects of this agent to induce slow wave sleep<sup>45</sup> (Table 1). Furthermore, the hypnotic effects of etomidate, as assessed by the loss of the righting reflex (LORR), were influenced by this  $\beta$ 2 mutation (Table 1). Complementary studies have been were conducted in a  $\beta$ 3N265M mouse. Note, aAs described above, *in vitro* studies revealed this methionine mutation to suppress the GABA-modulatory actions of etomidate, but to additionally blunt those of propofol. In  $\beta$ 3N265M mouse the sedative effects of etomidate were similar to those of the wild-type mouse<sup>10 44 46</sup>. Therefore, GABA<sub>A</sub>Rs containing the  $\beta$ 2 subunit mediate the sedative effects of this anaesthetic etomidate. However, the hypnotic effects (the duration of the LORR) of etomidate were reduced by the  $\beta$ 3 mutation. Collectively, these results suggest the hypnotic effects of etomidate to involve both  $\beta$ 2- and  $\beta$ 3-containing GABA<sub>A</sub>Rs (Table 1). The immobilising effect of etomidate, assessed by the hind limb withdrawal reflex was abolished (Table 1)<sup>10 44 46</sup>.

#### *The thalamo-cortical pathway – a site of action for intravenous general anaesthetics.*

The neuroanatomical substrates of general anaesthetic action remain elusive, although electrophysiological, neuroimaging and circuit-modelling studies consistently implicate the thalamus as an important locus for anaesthetic-induced sedation and hypnosis<sup>10 47–51</sup>. Indeed, the thalamus has a recognised the role in controlling conscious state transitions<sup>52</sup>. We

employed the whole-cell voltage-clamp technique to record from thalamic brain slices obtained from wild-type and GABA<sub>A</sub>R-mutant mice ~~the influence of etomidate on neural inhibition~~. Using GABA<sub>A</sub>R subtype selective drugs, immunohistochemistry and a variety of GABA<sub>A</sub>R mutant mice we determined that the synaptic GABA<sub>A</sub>Rs of the mouse thalamocortical ~~(TC)-ventrobasal (VB)~~ relay neurons are composed  $\alpha 1$ ,  $\beta 2$  and  $\gamma 2$  subunits<sup>53</sup> (Figure 3). Upon ~~the~~ vesicular release of GABA these receptors are briefly activated, resulting in the near simultaneous opening of a population of ~~associated~~ anion channels and the movement chloride ions, usually into the neuron, to cause phasic inhibition (an inhibitory post-synaptic potential, IPSP). Under voltage-clamp conditions, activation of synaptic GABA<sub>A</sub>Rs by GABA released from a single vesicle, results in a phasic miniature inhibitory postsynaptic current (mIPSC) – Figure 3. In response to a presynaptic action potential the near synchronous release of GABA from multiple vesicles produces an inhibitory postsynaptic current (IPSC). In wild-type mice low micromolar concentrations of etomidate greatly prolonged ~~the~~ mIPSC duration, whereas this effect was blunted in the  $\beta 2N265S$  mouse<sup>53 54</sup>. These ~~VB-ventrobasal~~ neurons additionally express peri- or extra-synaptic GABA<sub>A</sub>Rs, composed of  $\alpha 4$ ,  $\beta 2$  and  $\delta$  subunits that mediate a tonic form of inhibition<sup>53 54</sup> (Figure 3). In wild-type mice etomidate greatly increased tonic inhibition, but this effect was blunted in the  $\beta 2N265S$  mouse<sup>54</sup>. Therefore, the effects of etomidate to enhance both phasic and tonic inhibition are compromised in the  $\beta 2N265S$  mouse.

To better understand the relative importance of these effects of etomidate we first determined under more physiological conditions how phasic and tonic inhibition integrate to influence ~~VB ventrobasal~~ neuron excitability. ~~The VB-ventrobasal~~ neurons are innervated by a band of GABA-ergic nucleus reticularis ~~(nRT)-neurons, which that~~ provide the major source of inhibition. The ~~VB-ventrobasal~~ and the ~~nucleus reticularis nRT~~ neurons exhibit both burst and tonic firing modes, with tonic firing dominating during waking and burst firing during periods of drowsiness and NREM sleep<sup>55 56</sup>. Using paired recordings of synaptically coupled ~~nucleus reticularis nRT-VB-ventrobasal~~ neurons we demonstrated that high frequency burst firing of ~~the nucleus reticularis nRT~~ neurons produced a greatly prolonged IPSC that resulted from GABA activating synaptic  $\alpha 1\beta 2\gamma 2$  receptors, but additionally from the “spill-over” of GABA from the synapse, which then activated the extra-, or peri-synaptic  $\alpha 4\beta 2\delta$  GABA<sub>A</sub>Rs<sup>57</sup> (Figure 3). In support, ~~the~~ IPSC duration was reduced in equivalent recordings made from a mouse where the  $\alpha 4$  subunit was genetically deleted ( $\alpha 4^{-/-}$ )<sup>57</sup>. Furthermore, during burst firing, DS2, a  $\delta$ -GABA<sub>A</sub>R-selective PAMmodulator, greatly prolonged ~~the VB-ventrobasal~~ neuron IPSC duration derived from wild-type mice, but not those of the  $\alpha 4^{-/-}$  mouse<sup>57</sup>. Etomidate too greatly prolongs such

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IPSCs resulting from activation of synaptic, ~~and-but~~ particularly extrasynaptic GABA<sub>A</sub>Rs<sup>58</sup>. In complementary experiments, slow IPSCs in response to ~~nucleus reticularis ART~~ burst firing were still evident in thalamocortical neurones, even though their synaptic  $\alpha 1$ -GABA<sub>A</sub>Rs had been genetically deleted<sup>59</sup>. Furthermore, although lacking synaptic  $\alpha 1$ -GABA<sub>A</sub>Rs *in vivo* thalamic recordings from such mice still revealed slow oscillations, or sleep spindles<sup>59</sup>.

A recent *in vivo* study further highlights the importance of these thalamic extrasynaptic GABA<sub>A</sub>Rs to the behavioural effects of etomidate. An increase in frontal electro-cortical activity in the  $\alpha$ - $\beta$  frequency range is considered to signal an anaesthetic-induced loss of consciousness<sup>60</sup>. Elevated thalamic  $\alpha$ - $\beta$  activity precedes similar activity in cortex<sup>61</sup>. During non-rapid-eye-movement (NREM) sleep, *i.e.* a state associated with burst firing, microperfusion of etomidate directly into the thalamus increased  $\alpha$ - $\beta$  activity of wild-type, but not that of mice where the  $\delta$  subunit had been genetically deleted ( $\delta^{-/-}$  mice)<sup>60</sup>. Furthermore, these effects were mimicked by microperfusion of DS2, a  $\delta$ -GABA<sub>A</sub>R selective ~~PAM-modulator~~<sup>60</sup>. Collectively, these studies further highlight the thalamus as a critical locus for anaesthetic action, and in particular emphasise the importance of the effect of agents such as etomidate to enhance spill-over inhibition. The pharmacology of the thalamic synaptic and extrasynaptic receptors and their differential impact upon brief phasic, tonic and spill-over inhibition is provided in Table 3. Note  $\delta$ -GABA<sub>A</sub>Rs are insensitive to benzodiazepines. Therefore, in these thalamocortical relay neurones agents such as diazepam and midazolam will prolong brief phasic inhibition mediated by synaptic GABA<sub>A</sub>Rs ( $\alpha 1\beta 2\gamma 2$ ), with no effect on tonic inhibition mediated by extrasynaptic GABA<sub>A</sub>Rs ( $\alpha 4\beta 2\delta$ ). ~~They~~ ~~only~~ exert a limited impact upon the “spill-over” IPSCs occurring during burst firing, which although involving synaptic GABA<sub>A</sub>Rs, are dominated by the contribution of the  $\delta$ -GABA<sub>A</sub>Rs.

#### *The role of GABA<sub>A</sub>Rs in the impairment of cognition associated with intravenous general anaesthetics-*

Short term impairment of memory is an important property of general anaesthetics, however, clinically their use and the accompanying surgery ~~may-can~~ be associated with a post-operative cognitive impairment, lasting for days to months after administration<sup>62</sup>. The hippocampus is known to play an important role in the processes associated with both learning and memory. Hippocampal CA1 pyramidal neurones express extrasynaptic receptors composed of  $\alpha 5$ ,  $\beta$  and  $\gamma 2$  subunits that mediate tonic inhibition<sup>63</sup>. This tonic conductance is not evident in CA1

neurones derived from  $\alpha 5^{-/-}$  mice<sup>63</sup>. Such tonic currents are enhanced by general anaesthetics such as etomidate<sup>64</sup> and ~~were are~~ reduced by ~~the~~ subsequent co-application of L-655,708, a selective negative allosteric modulator (~~NAM~~) of  $\alpha 5\beta\gamma 2$  GABA<sub>A</sub>Rs<sup>64</sup>. Hippocampal long-term potentiation (LTP), a form of synaptic plasticity associated with learning and memory, is suppressed by etomidate in CA1 neurones obtained from wild-type but not from  $\alpha 5^{-/-}$  mice<sup>65</sup>. Furthermore, ~~the~~ suppression of LTP by etomidate was prevented by co-application of L-655,708<sup>64 65</sup>. Behaviourally, spatial and non-spatial hippocampal-dependant learning tasks were impaired by prior etomidate treatment, a deficit also prevented by treatment with L-655,708<sup>64 65</sup>.

Intriguingly, ~~the~~ administration of sedative and anaesthetic doses of etomidate impaired memory performance in the novel object recognition (NOR) test for over 3 to 7 days, respectively, ~~before recovery~~. Implicating  $\alpha 5$ -GABA<sub>A</sub>Rs, this impairment did not occur in the  $\alpha 5^{-/-}$  mouse, and was prevented by treatment with the  $\alpha 5$ -GABA<sub>A</sub>R ~~NAM-invers~~ agonist L-655,708<sup>66</sup>. How does etomidate produce such a prolonged effect on cognition, maintained long after ~~the~~ elimination of the anaesthetic? In tandem with these behavioural impairments, *ex vivo* recordings from CA1 neurones of mice previously administered etomidate revealed a persistent increase of the CA1 neurone tonic current mediated by  $\alpha 5$ -GABA<sub>A</sub>Rs and an associated decrease in the magnitude of LTP<sup>66</sup>. Complementary biochemical experiments revealed ~~an~~ increased cell surface expression of  $\alpha 5$ -GABA<sub>A</sub>Rs in the hippocampus. The mechanism of this anaesthetic-induced plasticity is not known. However, this effect of etomidate could be replicated in hippocampal cell cultures and required the presence of astrocytes, which presumably release some as yet unknown factor<sup>66</sup>. A possible candidate is the inflammatory cytokine interleukin- $1\beta$  (IL- $1\beta$ ). Inflammation triggered by surgical trauma and/or by the anaesthetic *per se* may increase circulating levels of IL- $1\beta$ <sup>67</sup>. In the hippocampus, this cytokine has been shown to increase cell surface expression of  $\alpha 5$ -GABA<sub>A</sub>Rs and to consequently increase ~~the~~ CA1 tonic current<sup>67</sup>. Indeed, etomidate and IL- $1\beta$  act synergistically to greatly enhance ~~the~~ CA1 tonic current mediated by  $\alpha 5$ -GABA<sub>A</sub>Rs<sup>68</sup>.

### Conclusions

~~We have summarised evidence that t~~The GABA<sub>A</sub>R is an important target for ~~mediating~~ the behavioural actions of general anaesthetics such as etomidate and propofol. Mammals express 20-30 different GABA<sub>A</sub>R isoforms. These receptor subtypes are not uniformly expressed throughout the CNS, but exhibit specific expression profiles, not only within different regions of

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the CNS, but even within individual neurones. ~~Therefore,~~ ~~g~~Given this very specific neuroanatomical expression profile, ~~in retrospect~~ it is perhaps not surprising that specific GABA<sub>A</sub>R subtypes mediate distinct components of the behavioural repertoire of drugs such as benzodiazepines, etomidate and propofol. The use of genetic techniques that permit the manipulation of wild-type and or mutant GABA<sub>A</sub>Rs in a neuron~~al~~-specific manner, coupled with advances in optogenetic manipulations, should further develop our understanding of the neuronal circuitry implicated in these drug-induced behaviours. The use of mice engineered to express anaesthetic-insensitive GABA<sub>A</sub>Rs has identified the molecular targets for developing novel therapeutics, with a reduced propensity for side effects. The challenge now is for the medicinal chemists to exploit the molecular differences between the various GABA<sub>A</sub>R isoforms to develop a new generation of medicines.

#### Author Contributions

—All authors contributed equally to the preparation of the manuscript, tables and figures.

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#### Declaration of Interests

CJW was a member of the editorial board of BJA (~~Education (formally Continuing Education in Anaesthesia Critical Care and Pain)~~ 2007-2017).

SJM —~~None declared~~and

JJL —~~None declared~~have no conflicts to declare.

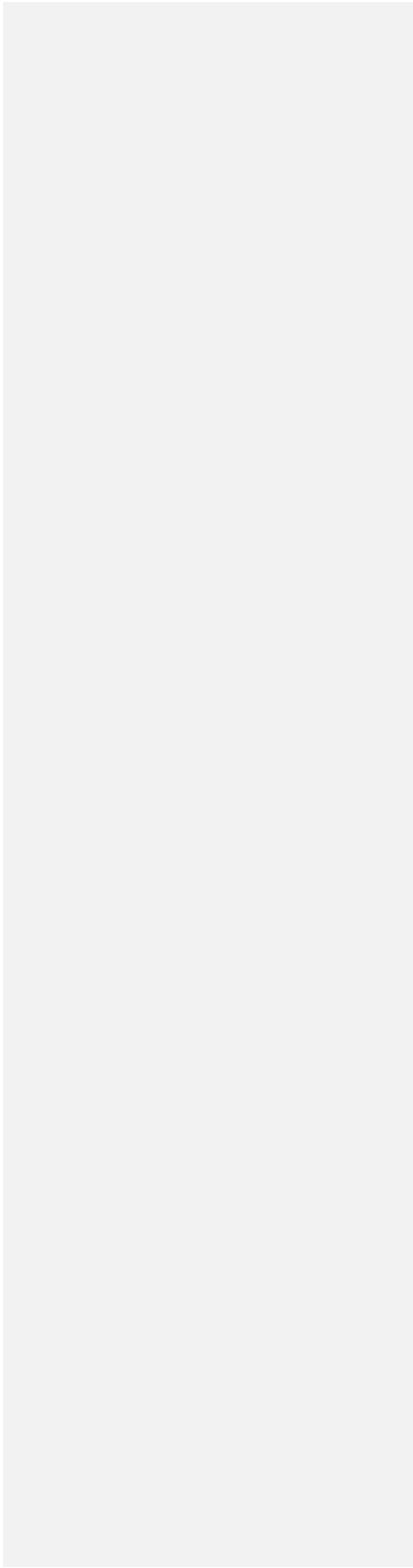
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<i>Drug</i>	<i>GABA<sub>A</sub>R subtype</i>	<i>Behaviour influenced</i>
Benzodiazepine	$\alpha 1\beta\gamma 2$	Sedation Anterograde amnesia Anticonvulsant actions
Benzodiazepine	$\alpha 2\beta\gamma 2$	Anxiolysis Analgesia Myorelaxation
Benzodiazepine	$\alpha 3\beta\gamma 2$	Analgesia Myorelaxation
Benzodiazepine	$\alpha 5\beta\gamma 2$	Cognitive impairment Myorelaxation
Etomidate	$\beta 2$ -GABA <sub>A</sub> Rs	Sedation Hypnosis
Etomidate	$\beta 3$ -GABA <sub>A</sub> Rs	Hypnosis Immobility

Table 1. The behavioural repertoire of benzodiazepines and ~~that~~ of the intravenous general anaesthetic etomidate are mediated by different GABA<sub>A</sub>R isoforms. Modified from <sup>23</sup>

<b>Drug</b>	<b>Phasic inhibition</b>	<b>Tonic inhibition</b>	<b>"Spill-over" inhibition</b>
Benzodiazepine	Enhanced	No effect	Modest enhancement
Neuroactive steroid	Enhanced	Enhanced	Enhanced
Etomidate	Enhanced	Enhanced	Enhanced
Propofol	Enhanced	Enhanced	Enhanced
Pentobarbitone	Enhanced	Enhanced	Enhanced
THIP	No effect	Enhanced	Little/no effect
DS2	No effect	Enhanced	Enhanced

*Table 2. The pharmacology of thalamic inhibition.* Thalamic relay neurons exhibit three kinetically distinct forms of GABA<sub>A</sub>R-mediated inhibition—(see Figure 3): 1) rapid phasic inhibition mediated by synaptic  $\alpha 1\beta 2\gamma 2$  GABA<sub>A</sub>Rs; 2) persistent tonic inhibition mediated by extrasynaptic  $\alpha 4\beta 2\delta$  GABA<sub>A</sub>Rs, and 3) at relatively high frequencies of presynaptic stimulation GABA spills over from the synapse to additionally activate extrasynaptic, or perisynaptic GABA<sub>A</sub>Rs, producing a prolonged albeit phasic composite—"spillover" inhibitory postsynaptic current (IPSC), with a minor contribution from synaptic GABA<sub>A</sub>Rs, but a dominant contribution from extrasynaptic GABA<sub>A</sub>Rs. Etomidate enhances all forms of inhibition, whereas DS2, a  $\delta$ -GABA<sub>A</sub>R-selective PAM, enhances tonic and spillover inhibition only, with no effect on fast phasic inhibition<sup>57 58</sup>. By contrast, THIP is a  $\delta$ -GABA<sub>A</sub>R agonist that has little effect on fast phasic inhibition, but increases tonic inhibition and is predicted to have little impact on spillover inhibition<sup>53 54</sup>. As pentobarbitone, propofol, and the neuroactive-steroidal anaesthetic alphaxalone, in common with etomidate are known to act as PAMs of both synaptic GABA<sub>A</sub>Rs and extrasynaptic  $\delta$ -GABA<sub>A</sub>Rs, they are predicted to additionally greatly enhance "spillover" inhibition. By contrast, benzodiazepines, such as diazepam or midazolam have no

effect on  $\delta$ -GABA<sub>A</sub>Rs and therefore are predicted to have only a modest influence on “spillover” inhibition. Importantly, during non-rapid-eye-movement (NREM) sleep, which is associated with burst firing, microperfusion of etomidate, or DS2 directly into the thalamus, increased  $\alpha$ - $\beta$  activity of wild-type, but not that of  $\delta^{-/-}$  (knockout) mice<sup>60</sup>.

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## Figure legends

**Figure 1:** *General anaesthetics are chemically diverse.* Shown are the chemical structures of representative intravenous and inhalational general anaesthetic agents. Although chemically disparate, ~~in common~~ all of these anaesthetics act as ~~PAMs~~ positive allosteric modulators of the GABA<sub>A</sub>R. ~~Additionally, at~~ greater concentrations they directly activate the GABA<sub>A</sub>R. ~~The A~~ schematic GABA<sub>A</sub>R ~~is~~ shown ~~is~~ composed of two  $\alpha$ , two  $\beta$  and a  $\gamma$  subunit, a common subunit stoichiometry for a synaptic GABA<sub>A</sub>R.

**Figure 2:** *The GABA<sub>A</sub>R is a target for certain intravenous general anaesthetics.* The effects of ~~four~~ 4 chemically distinct intravenous general anaesthetics on recombinant receptors expressed in *Xenopus laevis* oocytes ~~was are determined shown~~ <sup>36 37</sup>. The arrows pointing up and down indicate enhancement; or inhibition ~~respectively~~ of the agonist-evoked response. The number of arrows indicates the magnitude of the effect, e.g. 3 upward arrows indicates a large ~~potent~~ enhancement of the response, ~~3 downward arrows indicates a potent inhibitory effect of the anaesthetic.~~ ~~Note that at~~ behaviourally relevant concentrations all anaesthetics greatly enhance the GABA-evoked response. The red star (\*) indicates that in common with alphaxalone, betaxalone, ~~the a~~ behaviourally inert 3 $\beta$ -ol isomer, ~~inhibits~~ nAChR receptors. See <sup>9</sup>

**Figure 3** *Thalamic relay neurones exhibit phasic, tonic and spill-over inhibition mediated by GABA<sub>A</sub>Rs.* **A)** ~~The~~ vesicular release of GABA (red dots and cloud) causes activation of synaptic (blue ~~receptors~~)  $\alpha 1\beta 2\gamma 2$  GABA<sub>A</sub>Rs resulting in ~~a~~ fast transient ~~form of~~ phasic inhibition, the miniature inhibitory postsynaptic current (mIPSC), ~~(resulting from~~ GABA released from a single vesicle (~~—see trace on the right~~), or following a presynaptic action potential an IPSC (produced by multi-synchronous vesicular release). **B)** These neurones additionally express extrasynaptic  $\alpha 4\beta \delta$  GABA<sub>A</sub>Rs, (orange receptors), which are activated by relatively low ambient concentrations of GABA, resulting in a persistent tonic form of inhibition. The trace on the left shows, on a relatively slow time scale, the holding current in control and after the application of the GABA<sub>A</sub>R antagonist bicuculline, which produces an outward current and a decrease in the membrane noise as the extrasynaptic GABA<sub>A</sub>Rs are ~~closed~~ blocked by the antagonist. Note the



downward spikes represent ~~the~~ miniature IPSCs, which are also abolished by bicuculline blocking ~~the~~ synaptic GABA<sub>A</sub>Rs. **C**). High frequencies of presynaptic action potentials (e.g. that occur during burst firing) cause substantial spill over of GABA from the synapse (represented by the increased red cloud), which then activates ~~the~~ extrasynaptic GABA<sub>A</sub>Rs, to produce a prolonged slow form of phasic inhibition. Note the increased and prolonged IPSC relative to synaptic inhibition (red shaded area). See <sup>57 58</sup>

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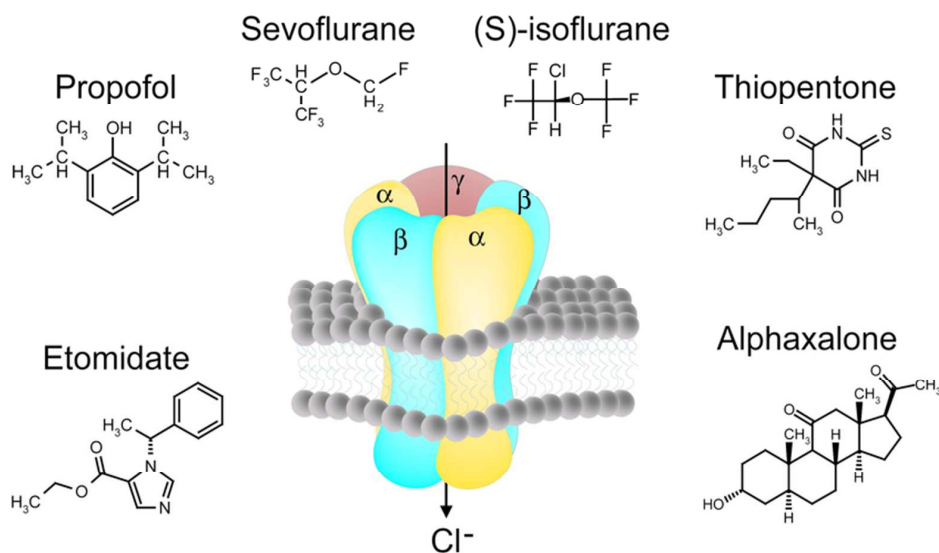


Figure 1: General anaesthetics are chemically diverse. Shown are the chemical structures of representative intravenous and inhalational general anaesthetic agents. Although chemically disparate, in common all of these anaesthetics act as PAMs of the GABA<sub>A</sub>R. Additionally, at greater concentrations they directly activate the GABA<sub>A</sub>R. The schematic GABA<sub>A</sub>R shown is composed of two α, two β and a γ subunit, a common subunit stoichiometry for a synaptic GABA<sub>A</sub>R.

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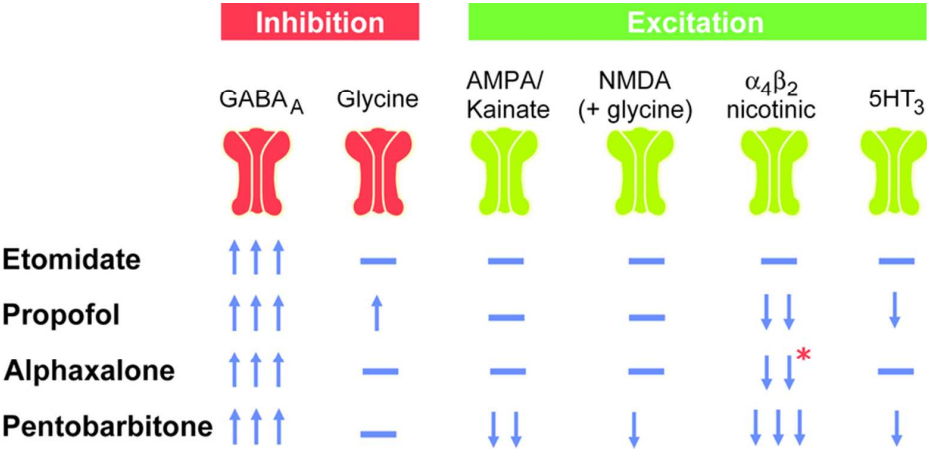


Figure 2: The GABA<sub>A</sub>R is a target for certain intravenous general anaesthetics. The effects of 4 chemically distinct intravenous general anaesthetics on recombinant receptors expressed in *Xenopus laevis* oocytes was determined<sup>36 37</sup>. The arrows pointing up and down indicate enhancement, or inhibition respectively of the agonist-evoked response. The number of arrows indicates the magnitude of the effect e.g. 3 upward arrows indicates a large potent enhancement of the response, 3 downward arrows indicates a potent inhibitory effect of the anaesthetic. Note that at behaviourally relevant concentrations all anaesthetics greatly enhance the GABA-evoked response. The red star (\*) indicates that in common with alphaxalone, betaxalone, the behaviourally inert 3β-ol isomer inhibits nAChR receptors. See<sup>9 46</sup>

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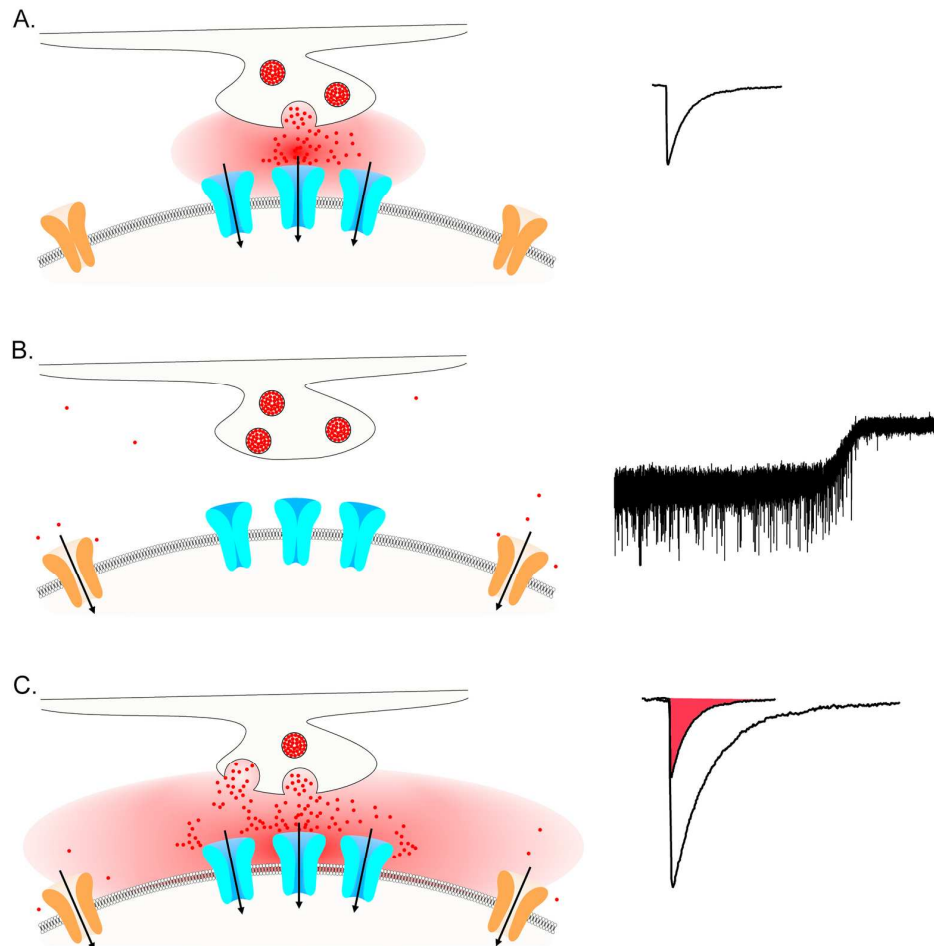


Figure 3 Thalamic relay neurons exhibit phasic, tonic and spill-over inhibition mediated by GABA<sub>A</sub>Rs. A) The vesicular release of GABA (red dots and cloud) causes activation of synaptic (blue receptors)  $\alpha 1\beta 2\gamma 2$  GABA<sub>A</sub>Rs resulting in a fast transient form of phasic inhibition, the mIPSC (resulting GABA released from a single vesicle – see trace on the right), or following a presynaptic action potential an IPSC (produced by multi-synchronous vesicular release). B) These neurons additionally express extrasynaptic  $\alpha 4\beta\delta$  GABA<sub>A</sub>Rs, (orange receptors), which are activated by relatively low ambient concentrations of GABA, resulting in a persistent tonic form of inhibition. The trace on the left shows, on a relatively slow time scale, the holding current in control and after the application of the GABA<sub>A</sub>R antagonist bicuculline, which produces an outward current and a decrease in the membrane noise as the extrasynaptic GABA<sub>A</sub>Rs are closed by the antagonist. Note the downward spikes represent the mIPSCs, which are also abolished by bicuculline blocking the synaptic GABA<sub>A</sub>Rs. C). High frequencies of presynaptic action potentials (e.g. that occur during burst firing) cause substantial spill over of GABA from the synapse (represented by the increased red cloud), which then activates the extrasynaptic GABA<sub>A</sub>Rs, to produce a prolonged slow form of phasic inhibition. Note the increased and prolonged IPSC relative to synaptic inhibition (red shaded area). See <sup>57 58</sup>

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